The Uptake of 5-Hydroxytryptamine by *Litomosoides carinii* and the Effect of Antifilarials

J. K. SAXENA, Ramanuj SEN, R. K. CHATTERJEE* and S. GHATAK

*Divisions of Biochemistry and Parasitology**
Central Drug Research Institute, Lucknow-226001, India

Abstract: *Litomosoides carinii*, the filarial parasite of cotton rat (*Sigmodon hispidus*), has been shown to possess both high and low affinity uptake mechanisms for 5-hydroxytryptamine (5-HT). The Michaelis-Menten constant (K_m) and V_{max} values for high affinity system were 1.9 μM and 1.25 μM/g/2 min, while the corresponding values for the low affinity system were 10 μM and 6.6 μM/g/2 min respectively. The uptake mechanism was found to be temperature dependent and was inhibited by microfilaricides-diethylcarbamazine and centperazine, the latter exhibiting more pronounced effect. The filarial worms could not synthesize 5-HT from its precursor amino acid, tryptophan.

Key words: *Litomosoides carinii*, 5-hydroxytryptamine, Uptake mechanisms, Diethylcarbamazine, Centperazine, Synthesis

INTRODUCTION

Among the various criteria for an endogenously occurring neuroactive molecule to be designated as a neurotransmitter or a neuromodulator is the presence of a mechanism for its reuptake into nerve endings from the site of its release or action, thus contributing to the termination of its effect (Kannengiesser *et al.*, 1973; Koe, 1976). Such uptake mechanisms have been found to be specific and energy dependent processes which are sensitive to metabolic inhibitors, temperature, ion concentration and a variety of psychopharmacological agents (Shore, 1972; Kannengiesser *et al.*, 1973; Iverson, 1974; Garey, 1976). Studies from several laboratories have well established the existence of such uptake mechanisms for 5-hydroxytryptamine (5-HT) in a few parasitic worms (Bennett and Bueding, 1973; Hariri, 1975). Kinetic analysis has shown that the uptake mechanism is mediated through an active transport process, similar to that reported for a variety of brain tissue and synaptosomal preparations from vertebrate species (Shaskan and Snyder, 1970; Carlsson, 1970; Hamberger and Tuck, 1973; Wong *et al.*, 1973; Ross and Renyi, 1967, 1975). A previous communication from this laboratory had indicated the presence of several neuroamines both in microfilariae and adults of *Litomosoides carinii* (Saxena *et al.*, 1977). The present report describes the presence of an uptake system for 5-HT and the effect of a few filaricides on the uptake process in *L. carinii* adults as well as absence of a biosynthetic pathway for this neuroamine.

Received for Publication July 21, 1987.
CDRI Communication No. 3667.

MATERIALS AND METHODS

Parasites:

Adult, motile *L. carinii* were obtained from heavily infected cotton rats as described earlier (Saxena *et al.*, 1977). After removal from the host, the worms were thoroughly washed with normal saline (0.85% NaCl, w/v), immediately placed in Kreb's Ringer bicarbonate (KRB) medium (DeLuca and Cohen, 1964), adjusted to pH 7.4. All the chemicals used during the study were of analytical grade.

Incubations, regardless of temperature, were carried out in a Dubnoff metabolic shaking incubator (Precision Scientific, USA) adjusted at 50 strokes/min.

Measurement of uptake:

Prior to the addition of ^{14}C-5-HT (Amersham UK), the worms were preincubated in KRB medium at the specified temperature for 15 min to attain the equilibrium. After appropriate incubation time in presence of labelled 5-HT, the worms were removed from the incubation medium and flushed with ice-cold KRB for removing radioactivity adhering on worm surface. The worms were then blotted, weighed and homogenized in a chilled Potter Elvehjem tissue grinder containing 2 ml ice-cold perchloric acid (PCA, 0.4N). The homogenate was then centrifuged at 800 *g* for 10 min. For the determination of labelled 5-HT in the incubation medium, one ml aliquot was placed in a centrifuge tube containing an equal volume of 0.8 N PCA and centrifuged at identical speed and time. One ml portion each of *L. carinii* supernate and the incubation medium supernate were then placed in counting vials containing 15 ml scintillation cocktail for radioactivity measurements using a Packard Tricarb Scintillatoin counter. The scintillation fluid consisted of 0.4% (w/v) 2,5-diphenyloxazole and 0.04% (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in a mixture (2:1) of toluene and Triton X-100. Total uptake was calculated for 2 ml of both *L. carinii* supernate and incubation medium supernate. The amount of 5-HT entering the worms by passive diffusion and nonspecific binding was determined by incubation of the worms at 4 ℃. For kinetic studies, active transport was taken as the difference between the uptake measured at 37℃ and 4℃.

Existence (if any) of biosynthetic pathway for 5-HT was measured by incubating motile worms with tryptophan and pargyline (monoamine oxidase inhibitor) for different time intervals in KRB medium and then measuring 5-HT content of the worms according to Maickel *et al.* (1968) based on the formation of highly fluorescent products with o-phthalaldehyde (OPT) (Maickel and Miller, 1966).

RESULTS

The time-course for the uptake of 5-HT by *Litomosoides carinii* adults is depicted in Fig. 1. It was found that initially 5-HT incorporation was rapid and the uptake mechanism operated against a concentration gradient, suggesting thereby the presence of a mechanism characteristic of active transport. The tissue to medium ratio of 5-HT after a period of 90 min incubation were 5.5 (1 μM), 4.2(5 μM) and 3.03(10 μM) (Table 1). Tissue to medium

ratio decreases with increasing concentration of 5-HT in the medium. At 1 to 4 μM concentration, 5-HT uptake in the parasite tended to level off after 30 min. On the other hand at 5 to 10μM concentration the level increased progressively up to 90 min (Fig. 1). Effect of temperature on ^{14}C-5-HT uptake presented in Table 2 indicates a marked reduction of the uptake process occurring at 4℃ which remained constant upto 30 min of incubation.

Fig. 2 shows the course of initial rate of 5-HT uptake by *L. carinii* using varying concentrations of 5-HT. In the presence of low external concentrations of 5-HT, rapid accelration of the uptake velocity occurred, which started declining when the external

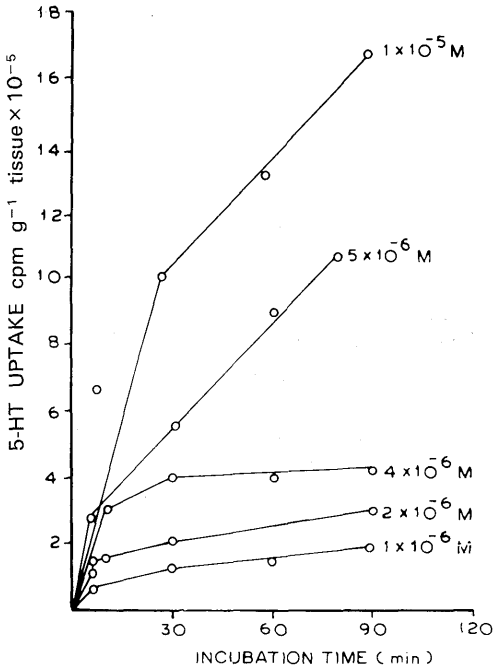

Fig. 1. Time course of 5-HT uptake by *L. carinii* at various concentrations of amine at 37℃.

Fresh, motile worms (100−200 mg) were incubated in KRB medium, pH 7.4, containing different concentrations of ^{14}C-5-HT at 37° for different time periods. The worms were removed from the medim, washed several time with the incubation medium, homogenized and counted for radioactivity.

Table 1. Uptake of ^{14}C-5-HT by *L. Carinii* at various concetration at 37℃

Concentration (μM)	Time (min)	Cpm g^{-1} tissue x10^{-5}	Cpm ml^{-1} medium x10^{-5}	Tissue to medium ratio
10	90	16.9	5.37	3.03
5	90	8.5	2.03	4.2
2	90	3.09	0.70	4.4
1	90	1.92	0.35	5.5

Number of experiments were four in each case.

concentration of 5-HT was around 1 μM and any increase in external concentration beyond this level resulted in a slow but linear increase.

Corresponding values of 5-HT concentration and its uptake when plotted according to the method of Lineweaver and Burk, showed the existence of two distinct K_m values indicating that the entry of 5-HT into *L. carinii* was mediated by both high and low affinity processes (Fig. 3). The K_m values for high affinity system was 1.9 μM whereas the low affinity system has a K_m value of 10 μM. The values for V_{max} for these systems were 1.25 and 6.6 μM/g/2 min respectively.

Table 3 shows the velocity of 5-HT uptake between 1 to 5 min at 25 and 37℃ with varying concentrations of 5-HT. The uptake was not proportional with different time intervals at 37℃ and the initial rate showed a similar pattern when the temperature was

Table 2. Effect of temperature on ^{14}C-5-HT uptake by *L. Carinii*.

Incubation time (min)	Concentration (μM)	5-HT uptake (Cpm g^{-1} tissue) $\times 10^{-4}$	
		37℃	4 ℃
5	5	6.95 (2)	2.17 (2)
30	5	10.94 (3)	2.35 (2)

Number of experiments are shown in parentheses.

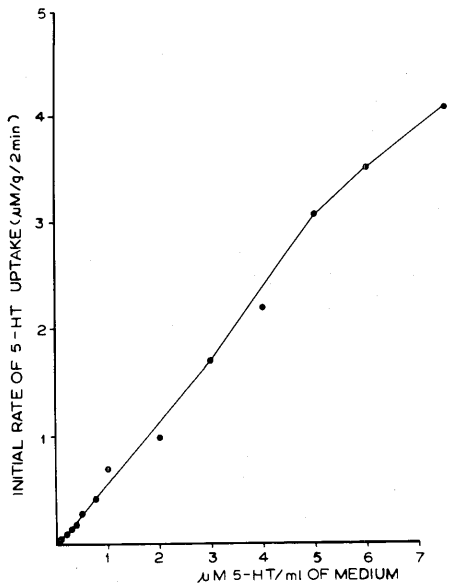

Fig. 2. Effect of external substrate concentration on initial rate of 5-HT uptake by *L. carinii* at 37℃. Specific uptake was taken as difference between the uptake at 37° and 0℃.

lowered to 25℃. The uptake after 5min at 25℃ was three times lower as compared to the values observed at 37℃.

Table 4 depicts the effect of some antifilarials on the 5-HT uptake. Centperazine, a filaricide introduced by CDRI, was found to be more effective in inhibiting 5-HT uptake in comparison to the well known antifilarial drug diethylcarbamazine (DEC). At 10 μM concentration, centperazine inhibited the uptake process by 36%, while inhibition due to DEC was only 12%. Suramin exerted no significant inhibition at this concentration.

The ability of the worms to retain 5-HT was determined by measuring the rate at which the organism lost 5-HT following incubation of the parasite at 37℃ for 60 min with

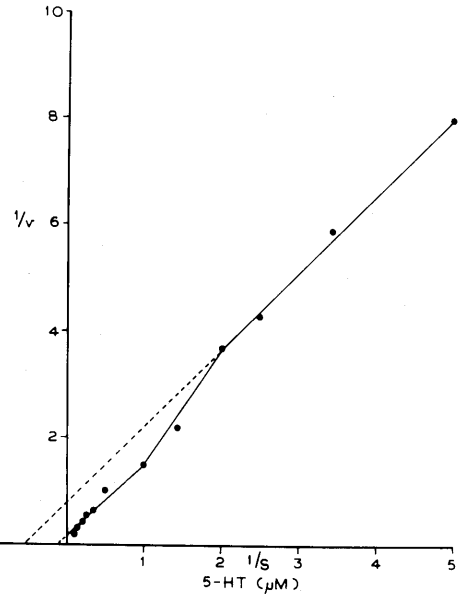

Fig. 3. Lineweaver-Burk plot of 5-HT uptake in 2 min incubation at 37℃.

Table 3. Velocity of ^{14}C-5-HT uptake in *L. carinii* measured at two temperatures, two concentrations and three time periods

Incubation time (min)	Concentration (μM)	Rate of uptake (cpm g^{-1} tissue) $\times 10^{-3}$ Temperature	
		25℃	37℃
1	0.2	0.77 (2)	2.42 (3)
2	0.2	2.63 (3)	3.23 (4)
3	0.2	3.37 (4)	ND
5	0.2	0.07 (3)	9.30 (3)
1	1.0	ND	21.02 (4)
2	1.0	ND	39.75 (4)
5	1.0	ND	42.10 (3)

Number of experiments are shown in parentheses.
ND=Not done.

1.0 and 0.2 μM 5-HT and subsequent incubation in 5-HT free medium for 30,60,120,150 and 180 min (Fig. 4). The release of 5-HT was found to be temperature dependent since no release was observed when the temperature was lowered to 4℃.

In order to find out the presence of a biosynthetic pathway for 5-HT in *L. carinii*, the worms were incubated with tryptophan and pargyline, a monoamine oxidase (MAO) inhibitor for different time intervals. The results presented in Table 5 showed that the parasite was not able to hydroxylate tryptophan and the motor activity of the worm remained unaffected.

Table 4. Effect of antifilarials on 5-HT uptake by *L. Carinii*

Drug	Concentration	Net uptake (Cpm g^{-1} tissue) $\times 10^{-4}$	Inhibition %
–	–	6.89 (3)*	–
Diethylcarbamazine	0.1 μM	6.6 (3)	4
	10.0 μM	6.05 (3)	12
	1.0 mM	5.12 (4)	20
Centperazine	0.1 μM	6.11 (2)	11
	10.0 μM	4.42 (4)	36
	1.0 mM	3.10 (4)	55
Suramin	10.0 μM	6.20 (2)	10

*Number of experiments are shown in parentheses.
Concentration of ^{14}C-5-HT was 1 μM.

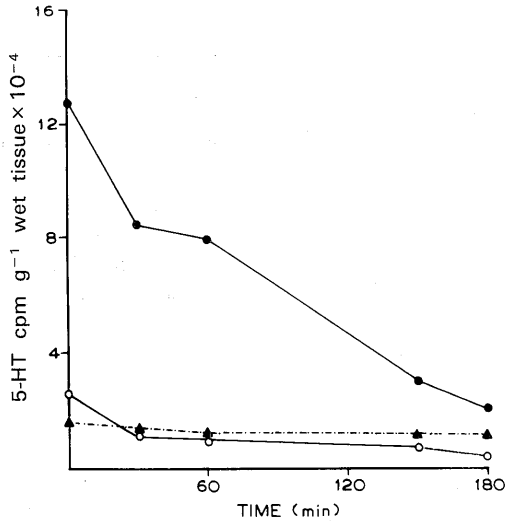

Fig. 4. Release of 5-HT by *L. carinii*.
Worms were preincubated for 1hr at 37℃ with 1×10^{-6}M 5-HT (●—●), 2×10^{-7}M 5-HT (○—○) and at 4℃ in 2×10^{-7}M 5-HT (▲—▲). The worms were then washed with incubation medium and transferred in the medium devoid of 5-HT for different time intervals.

Table 5. 5-HT levels of *L. carinii* following incubation at 37℃ in KRB medium with or without tryptophan (1×10^{-4}M) and monoamine oxidase inhibitor (1×10^{-3}M)

Addition	Incubation time (hrs)	5-HT (μg/g wet tissue)
None	3	0.411±0.06 (2)*
Tryptophan+ pargyline	3	0.401±0.09 (2)
None	6	0.252±0.05 (2)
Tryptophan+ pargyline	6	0.221±0.04 (3)
None	9	0.210±0.01 (2)
Tryptophan+ pargyline	9	0.196±0.06 (3)
None	12	0.241±0.08 (3)
Tryptophan+ pargyline	12	0.194±0.06 (3)

*Number of experiments are shown in parentheses.

DISCUSSION

Presynaptic neuronal uptake is one of the major mechanisms whereby biogenic amines are rendered pharmacologically inactive (Iverson, 1974). Bennett and Bueding (1973) demonstrated the 5-HT uptake in *Schistosoma mansoni* to be mediated by a saturable membrane transport system, while Hariri (1975) could not establish the functioning of such a system in *Mesocestoides corti*. Although, he pointed out that such a system could operate if the incubation time would have been prolonged. Thus, *Litomosoides carinii* having a 5-HT uptake mechanism which might not be mediated by a saturable membrane transport system, resembled *M. corti*. Similar uptake mechanism has been described for norepinephrine by brain slices and the cat heart (Dengler *et al.*, 1962) and for L-proline in *Hymenolopis diminuta* (Kilejian, 1966).

The reduction of 5-HT uptake at 4℃ in *L. carinii* suggested that beside an active uptake mechanism, the neuroamine was also entering the worm by a process of simple diffusion, similar to that observed in *S. mansoni* (Bennett and Bueding, 1973) and *M. corti* (Hariri, 1975).

Brain-slices and synaptosomes have specific transport system for nuerotransmitter amines (Iverson, 1970). A high affinity uptake process (K_m 0.17 μM) has been reported in brain slices (Shaskan and Synder, 1970) which is largely responsible for the transport of 5-HT at concentration below 1 μM. The high affinity for its substrate (5-HT) prevents the interference by catecholamines. The biphasic nature of 5-HT uptake observed in *L. carinii* at various external concentrations of this amine suggested that the mechanism of 5-HT

uptake was a complex process and at least two distinct transport systems are operating. At low concentration, an active transport system was operative, whereas at higher external concentration (greater than 0.5μM) 5-HT was probably being incorporated by a multiple transport system viz., simple diffusion plus an active transport system. Values of 5-HT concentration and its uptake plotted according to the method of Lineweaver and Burk (1934) clearly pointed the possibility of at least two distinct uptake mechanisms, the high affinity being specific for 5-HT and the low affinity uptake may involve catecholaminergic neurons. Sodium-dependent specific high affinity mechanism is associated with tryptaminergic neurons in mammalian brain (Kuhar, 1973). 5-HT is also taken up by dopaminergic and noradrenergic neuronal uptake sites, although with considerably lower affinity (K_m 8 μM) (Iverson, 1974). Accumulation of 5-HT by catecholaminergic neurons has been reported in vas deferens (Thoa *et al.*, 1969), pineal gland (Neff et al., 1969) and in brain (Lichtensteiger *et al.*, 1967). Bennett and Bueding (1973) have reported a K_m value for *S. mansoni* close to 0.5 μM, a value obtained by Shaskan and Snyder (1970) with rat brain slices. Hariri (1975) reported a K_m value in *M. corti* of 0.14 μM for high affinity uptake and 2.5 μM for low affinity system. Thus the filarial parasite *L. carinii* differed from *S. mansoni* and *M. corti* in its K_m values for 5-HT uptake. The differences in K_m values may possibly be due to a) different habitats and species of the parasites; b) due to different contents of 5-HT in them.

Iverson (1970) found that norepinephrine (NE) uptake in brain slices followed the classical Michaelis-Menten equation while in *L. carinii* the velocity was not proportional between 1 and 5 min at 37℃ and also at 25℃. These findings are similar to that reported in *S. mansoni* (Bennett and Bueding, 1973) and *M. corti* (Hariri, 1975).

5-HT uptake process is blocked by tricyclic antidepressants (Kannengiesser *et al.*, 1973; Rose and Renyi, 1975). Hycanthone treatment of *S. mansoni* infected mice significantly increased the *in vitro* uptake of 5-HT and its localization within the worm (Chou *et al.*, 1973; Bueding *et al.*, 1974). Among the antifilarials, centperazine was found to be more effective in inhibiting 5-HT uptake as compared to diethylcarbamazine, thereby suggesting that the mode of action of centperazine may possibly be due to interference with the metabolism of 5-HT. Centperazine has also been shown to inhibit certain metabolic processes of *Setaria cervi*, a bovine filarial parasite (Anwar *et al.*, 1978). ADP inhibits 5-HT uptake by altering distribution of Na^+/K^+ across the cell membrane (Drummond and Gordon, 1976) thus inhibition of 5-HT uptake by filaricides may be linked to a change in ADP concentration in *L. carinii.*

5-HT release in *L. carinii* was found to be temperature dependent. Similar findings were earlier reported for *S. mansoni* (Bennett and Bueding, 1973) and *M. corti* (Hariri, 1975). Although biosynthesis of 5-HT in mammalian brain has been well established (Gal *et al.*, 1963, 1974;Gal and Marshall, 1964; Levin *et al.*, 1960) evidence for *de novo* synthesis of 5-HT in *L. carinii* could not be presented, thus present findings are in agreement with those of Bennett and Bueding (1973) and Hariri (1975) in other helminths. However, negative evidence does not exclude the possibility of *de novo* systhesis of this neuroamine in *L. carinii.* The lack of increase in 5-HT level after prolonged incubation of the parasite with

tryptophan and a MAO inhibitor might merely reflect saturation of tryptophan hydroxylase with endogenous tryptophan. The occurrence of some other type of MAO, that can use 5-HT as substrate, but is not inhibited by conventional MAO inhibitor used, can not be ruled out. Three types of mitochondrial MAO capable of deaminating kynuramine are present in mouse, one type is harmine sensitive and the other (containing two forms) is harmine resistant (Squires, 1968).

The presence of low concentration of 5-HT and lack of tryptophan hydroxylase in adult stage of *L. carinii* indicate that the parasite must be obtaining their 5-HT from the host. The high affinity uptake mechanism in *L. carinii* might be responsible for the supply of this neurotransmitter which it may not be able to synthesize.

REFERENCES

1) Anwar, N., Srivastava, A. K. & Ghatak, S. (1978): Effect of antifilarials on the metabolic activity of *Setaria cervi*. Ind. J. Parasitol., **2**, 101−105.
2) Bennett, J. L. & Bueding, E. (1973): Uptake of 5-hydroxytryptamine by *Schistosoma mansoni*. Molecular Pharmacology **9**, 311−319.
3) Bueding, E., Bennett, J. L., Chou, T., Pert, C. & Tomosky, T. (1974): Effect of antischistosomal drugs on the uptake of 5-hydroxytryptamine by *Schistosoma mansoni*. Proc. 3rd Int. Cong. Prarasitol., **3**, 1443−1444.
4) Carlsson, A. (1970): A structural specificity for inhibition of ^{14}C-5-HT uptake by cerebral slices. J. Pharmac. Pharmacol., **22**, 729−732.
5) Chou, T. C., Bennett, J. L., Pert, C. T. & Bueding, E. (1973): Effect of hycanthone and of two of its structural analogs on levels and uptake of 5-hydroxytryptamine in *Schistosoma mansoni*. J. Pharmacol. Exp. Ther., **186**, 408−415.
6) DeLuca, H. F. & Cohen, P. P. (1964): In: Manometric techniques (4th eds) (ed. W. W. Umbreit, RH Burris and J. F. Stauffer), p. 132. Burgess Publishing Company USA.
7) Dengler, H. J., Michaelson, I. A., Speiegl, H. E. & Titus, E. (1962): The uptake of labelled norepinephrine by isolated brain and other tissues of the Cat. Int. J. Neuropharmacol., **1**, 23−38.
8) Drummond, A. H. & Gordon, J. L. (1976): Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by ADP. Brit. J. Pharmacol., **56**, 417−421.
9) Gal, E. M., Poezik, M. & Marshall, F. D. (Jr). (1963): Hydroxylation of tryptophan to 5-hydroxytryptophan by brain tissue *in vivo*. Biochem. Biophys. Res. Commun., **12**, 39−43.
10) Gal, E. M. & Marshall, F. D. (1964): The hydroxylation of tryptophan by pigeon brain *in vivo*. Progr. Brain Res., **8**, 56−60.
11) Gal, E. M., Morgan, M., Chatterjee, S. K. & Marshall, F. D. (1974): Hydroxylation of Tryptophan by brain tissue *in vivo* and related aspects of 5-hydroxytryptamine metabolism. Biochem. Pharmacol., **13**, 1639−1653.
12) Garey, R. E. (1976): Regional differences in the High affinity uptake of ^{3}H-dopamine and ^{3}H-norepinephrine in synaptosomes rich homogenates of cat brain. Life Sciences, **18**, 411−418.
13) Hamberger, B. & Tuck, J. R. (1973): Effect of tricyclic antidepressants on the uptake of NE and 5-HT by rat brain slices incubated in buffer and human plasma. Eur. J. Clin. Pharmacol., **5**, 1−7.
14) Hariri, M. (1975): Uptake of 5-hydroxytryptamine by *Mesocestoides corti* (Cestoda). J. Parasit., **61**, 440−448.

15) Iverson, L. L. (1970): In: Advances in Biochemical Pharmacology (Eds. E, Costa and E, Giacobini), Vol. 2, pp 109−132. Raven Press, New York.
16) Iverson, L. L. (1974): Uptake mechanisms for neurotransmitter amines. Biochem. Pharmacol., **23**, 1927−1935.
17) Kannengiesser, M. H., Hunt, P. & Raynaud, J. P. (1973): An *in vitro* model for the study of Psychotropic drugs and as a criterion of antidepressant activity. Biochem. Pharmacol., **22**, 73−84.
18) Kilejian, A. (1966): Permeation of L−proline in the cestode *Hymenolepis diminuta*. J. Parasit., **52**, 1108−1115.
19) Koe, B. K. (1976): Molecular Geometry of inhibitors of the uptake of catecholamines and Serotonin in synaptosomal preparations of rat brain. J. Pharmac. Exp. Ther., **199**, 649−661.
20) Kuhar, M. J. (1973): Neurotransmitter uptake: A tool in identifying neurotransmitter-specific pathways. Life Sciences., **13**, 1623−1634.
21) Levin, E. Y., Levenberg, B. & Kaufman, S. O. (1960): The enzymatic conversion of (3,4-dihydroxydroxyphenylethylamine) to norepinephrine. J. Biol. Chem., **235**, 2080−2086.
22) Lichtensteiger, W., Mutzner, U. & Langemann, H. (1967): Uptake of 5-hydroxytryptamine and 5-hydroxytryptophan by neurons of the central nervous system normally containing catecholamines. J. Neurochem., **14**, 489−497.
23) Lineweaver, H. & Burk, D. (1934): The determination of enzyme dissociation constants. J. Amer. Chem. Soc., **56**, 658−666.
24) Maickel, R. P. & Miller, F. P. (1966): Fluorescent products formed by reaction of indole derivatives and o−phthalaldehyde. Anal. Chem., **38**, 1937−1938.
25) Maickel, R. P., Cox, R. H (Jr)., Saillant, J. & Miller, F. P. (1968): A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. Int. J. Neuropharmacol., **7**, 275−281.
26) Neff, N. H., Barrett, R. E. & Costa, E. (1969): Kinetic and fluorescent histochemical analysis of the serotonin compartments in rat pineal gland. Eur. J. Pharmacol., **5**, 348−356.
27) Ross, S. B. & Renyi, A. L. (1967) Accumulation of tritiated 5-HT in brain slices. Life Sciences, **6**, 1407−1415.
28) Ross, S. B. & Renyi, A. L. (1975): Tricyclic antidepressant agents. I. Comparison of the inhibition of the uptake of ^{3}H-Noradrenaline and ^{14}C-5-HT in slices and crude synaptosome preparations of midbrain-Hypothalamus region of the rat brain. Acta Pharmacol. et. toxicol., **36**, 382−394.
29) Saxena, J. K., Bose, S. K., Sen, R., Chatterjee, R. K., Sen, A. B. & Ghatak, S. (1977): *Litomosoides carinii*: Biogenic amines in microfilariae and adults. Exp. Parasit., **43**, 239−243.
30) Shaskan, E. & Snyder, S. H. (1970): Kinetics of ^{3}H-5-hydroxytryptamine uptake into synaptosomes from different regions of the brain. Relation to catecholamine uptake. J. Pharmacol. Exp. Ther., **175**, 404−418.
31) Shore, P. A. (1972): Transport and storage of biogenic amines. Annu. Rev. Pharmac., **12**, 209−226.
32) Squires, R. F. (1968): Additional evidence for the existence of several forms of mitochondrial monoamine oxidase in the mouse. Biochem. Pharmacol., **17**, 1401−1409.
33) Thoa, N. B., Eccleston, D. & Axelrod, J. (1969): The accumulation of ^{14}C-serotonin in the guineapig vas deferens. J. Pharmacol. Exp. Ther., **169**, 68−73.
34) Wong, D. T., Horng, J. S. & Fuller, R. W. (1973): Kinctics of serotonin accumulation into synaptosomes of rat brain-Effects of amphetamine and chloroamphetamines. Biochem. Pharmacol., **22**, 311−322.